**Geographic variation and within-individual correlations of physiological stress markers in
a widespread reptile, the common garter snake (*Thamnophis sirtalis*)**

**Eric J. Gangloff^{a*}, Amanda M. Sparkman^b, Kaitlyn G. Holden^a, Caitlyn J. Corwin^a,
Madeline Topf^a, Anne M. Bronikowski^a**

^aDepartment of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, IA
50011, USA

^bDepartment of Biology, Westmont College, Santa Barbara, CA 93108, USA

Running title: Baseline and stress-induced physiology in garter snakes

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*Corresponding author

Eric J. Gangloff

Department of Ecology, Evolution, & Organismal Biology

Iowa State University

2200 Osborne Drive

251 Bessey Hall

Ames, IA 50011, USA

Tel: +1 515-708-0612

gangloff@iastate.edu

Abstract

Characterizing the baseline and stress-induced hormonal, metabolite, and immune profiles of wild animals is important to assess the impacts of variable environments, including human-induced landscape changes, on organismal health. Additionally, the extent to which these profiles are coordinated across physiological systems within individuals remains an important question in understanding how stressors can differentially affect aspects of an individual's physiology. Here, we present data from wild populations of the common garter snake (*Thamnophis sirtalis*) on both baseline and stress-induced biomarkers: plasma corticosterone (CORT) concentration, plasma glucose concentration, and whole blood heterophil:lymphocyte ratio. Using a standardized restraint protocol with individuals from populations in disparate portions of this species' range – the Sierra Nevada Mountains of California and the plains of Iowa – we collected blood plasma samples at nine time points over three days. Both CORT and glucose response curves differed between georegions, with Iowa snakes attaining higher glucose concentration and maintaining elevated CORT and glucose levels for a longer duration. Additionally, both the total amount and proportional increases of CORT and glucose were lower in larger and therefore older snakes, suggesting ontogenetic shifts in stress perception or response. Within-individual correlation among the three physiological indicators was significant at the time of capture, absent after 3 h in captivity, and partially restored after 3 d in captivity, demonstrating the effect of stress on the relationships among these physiological systems. Together, these results provide further evidence for the great physiological flexibility of ectothermic tetrapods in maintaining homeostasis across a range of factors.

Keywords: allostasis, corticosterone, geographic variation, glucose, homeostasis, hormones, leukocyte

1. Introduction

How physiological biomarkers of organismal energy status and homeostatic maintenance respond to stressors remains an important question with implications for both conservation (Baker et al., 2013; Busch and Hayward, 2009; Dantzer et al., 2014; Wikelski and Cooke, 2006) and evolution (Cox et al., 2016; Crespi et al., 2013; Dantzer et al., 2016; Palacios et al., 2012; Patterson et al., 2014). In both endotherms and ectotherms, organismal homeostasis is characterized by the physiological processes that hold constant the *milieu intérieur* with the purpose of maintaining steady state functionality across a range of external environments and internal conditions (Bradshaw, 2003; Chrousos, 1998). Recently, this concept has been expanded with the concept of allostasis, which incorporates variation in set points for internal variables in response to predictable or cyclical events and describes the energetic consequences (“allostatic load”) of deviation from this dynamic equilibrium (McEwen and Wingfield, 2010).

Glucocorticoids, hormones that mediate trade-offs among energetic allocations and maintain homeostasis in response to stressors, have been the primary subject of studies quantifying organismal responses to perturbations in homeostasis or allostasis (Angelier and Wingfield, 2013; Sapolsky et al., 2000; Wingfield et al., 1998). Additionally, much work has been done to estimate the correlation between both baseline and stress-induced levels of glucocorticoids and organismal fitness, though this relationship remains unclear (reviewed in Bonier et al., 2009; Breuner et al., 2008; Crespi et al., 2013; but see Baker et al., 2013). At both baseline stress-

induced levels, variation in glucocorticoids is driven by past experiences, current energetic status, reproductive status, and age (e.g. in snakes, Dayger et al., 2013; Lutterschmidt et al., 2009; Sparkman et al., 2014; reviewed in Sapolsky et al., 1986). As such, myriad factors must be taken into account when assessing the functional role of these hormones in wild animals, such as when comparing across populations (Eikenaar et al., 2012; Moore et al., 2001) or quantifying the ability of individuals to modulate their stress response (Wingfield, 2013). Higher baseline CORT concentration in wild animals is widely construed to indicate increased levels of physiological stress, but interpretation of CORT levels is not always straightforward. Recent work suggests that stress-induced CORT concentrations may reveal more about an organism's physiological ability to deal with stressors than do baseline levels (Breuner et al., 2008; Neuman-Lee et al., 2015; Vitousek et al., 2014). Both the magnitude and the duration of the stress response are important for assessing the functionality of hormonally-regulated stress response systems (Dickens and Romero, 2013; McCormick et al., 2015; Taff and Vitousek, 2016).

In reptiles, the neuroendocrine stress response is primarily facilitated by the hypothalamic–pituitary–interrenal (HPI) axis, with corticosterone (CORT) acting as the primary glucocorticoid (Norris and Jones, 1987). Glucocorticoid hormones modulate numerous day-to-day functions, including feeding, locomotor activity, energy metabolism, and immune function (reviewed in Landys et al., 2006). In response to a stressor, circulating glucocorticoids increase on an order of minutes to hours after the event and serve to promote recovery from stress and re-establish homeostasis (Bradshaw, 2003; Romero, 2004; Wingfield and Kitaysky, 2002). For both baseline and stress-induced measures, the effects of total plasma CORT may be modulated at multiple nodes, including the relative concentrations of binding globulin (reviewed

in Breuner et al., 2013) as well as receptor type and density (reviewed in Angelier and Wingfield, 2013; Landys et al., 2006; Lema and Kitano, 2013). Nonetheless, measures of total CORT concentration remain an important tool for assessing physiological status (Schoech et al., 2013), especially when measured at both baseline and stress-induced levels and in conjunction with other physiological stress biomarkers such as glucose and white blood cell counts (Breuner et al., 2013). After an encounter with a stressor, glucose concentrations are increased rapidly (within minutes) by actions of catecholamines. This is followed by increases in circulating glucocorticoids, which promote production of glucose via gluconeogenesis and glycogenolysis (Sapolsky et al., 2000). After the HPI axis has responded to a stressor, circulating glucose levels generally positively covary with the glucocorticoid response (e.g., Gangloff et al., 2016), providing opportunity to measure both the glucocorticoid stress response and its subsequent downstream effects. Additionally, glucocorticoids act to redistribute leukocytes to specific target tissues. For example, heterophil:lymphocyte (H:L) ratios generally increase in response to elevated glucocorticoids and remain stable over longer time scales (Goessling et al., 2015). However, despite their established functional relationship, H:L ratios and levels of circulating glucocorticoids are not always correlated within individuals (Goessling et al., 2015; Sparkman et al., 2014). The differential responses of physiological systems to both the intensity and duration of stressors thus underscores the need to use multiple markers when assessing physiological status (Breuner et al., 2013; Goessling et al., 2015).

In this study, we quantified multiple measures of both baseline and stress-induced physiological biomarkers in a widespread terrestrial vertebrate, the common garter snake (*Thamnophis sirtalis*). Previous work with this species demonstrates that glucocorticoids cycle daily and

seasonally and interact with other hormonal axes to regulate reproductive and feeding behaviors (Lutterschmidt and Maine, 2014; Lutterschmidt and Mason, 2008; Moore and Jessop, 2003; Moore et al., 2000a; Moore et al., 2000b; Moore and Mason, 2001) as well as life-history transitions (Cease et al., 2007). Furthermore, much work has been done on the short-term (< 4 h) glucocorticoid response of *T. sirtalis* from natural populations, mostly in relation to reproductive behaviors and trade-offs. For example, handling stress results in increased CORT concentrations and reduced testosterone concentrations in males during the reproductive season, but does not alter reproductive behavior (Moore et al., 2001; Moore et al., 2000a). The present study adds to the rich history of quantifying the hormonal stress response in this species by providing multiple measures of the stress response over a longer duration of a stressor. Data on the cumulative stress response over a number of days are important in interpreting stress indicators to assess individual or population-level health status.

Our study has three primary goals that complement previous work with this species: (1) to assess spatial variation in *T. sirtalis* physiological profiles by comparing multiple populations from disparate parts of this species' geographic range, (2) to test the relative roles of size, sex, reproductive condition, and time of year in determining both baseline patterns and stress-induced cumulative physiological response, and (3) to quantify the within-individual correlations among multiple physiological indicators (CORT, glucose, H:L ratios) across an extended stress response. By examining both the within-individual relationship of physiological indicators and multiple sources of among-individual variation, this study provides insight into the factors that affect the ability of individuals in natural populations to maintain functionality and energy balance in different ecological contexts.

2. Materials and Methods

2.1 Field Methods

We sampled multiple populations of *T. sirtalis* from each of two geographic regions (hereafter “georegions”) representing very different habitat types within the broad geographic range of this species, which extends across much of North America (Ernst and Ernst, 2003). The California populations are found in mountain meadows on federally managed land in largely undisturbed habitats (elevation 1600–2100 m). The Iowa populations represent a sampling of both natural areas and impacted sites in temperate grasslands (elevation 250–300 m). All snakes were located in the open or under cover objects and hand-captured at four sites in CA and four sites in IA (Table 1). We conducted fieldwork during daylight hours (between 7 30 h and 17 30 h; no difference between georegions: $t_{84} = -1.34$, $P = 0.18$) and during the active season (June 2013 in CA; May to October 2013 and 2014 in IA). Garter snakes generally mate immediately upon emergence from hibernation in the spring (Rossman et al., 1986), which for these populations occurs at least a month before our field observations. Furthermore, we did not observe any reproductive behaviors during our fieldwork, indicating that measures on these individuals were likely not conducted during mating season. Upon capture, we first measured snake body temperature using a cloacal thermometer (DeltaTrak FlashCheck Probe Thermometer, Pleasanton, CA, USA) and then immediately drew a baseline blood sample (50–150 μ L) from the caudal vein using a heparinized syringe. All blood samples for baseline measures were collected ≤ 10 min after time of capture, which is before CORT concentration is elevated due to handling stress in a congeneric species (*Thamnophis elegans*; Palacios et al., 2012).

For a subset of baseline samples from both georegions ($N = 53$), we used fresh blood to prepare blood smears, which we fixed with methanol and stained with Wright-Giemsa stain (Fisher Scientific Inc., Pittsburgh, PA, USA). We stored blood on ice for up to 3 h, then separated plasma by centrifugation, snap-froze in liquid nitrogen, and stored at -80°C until the time of assays. After blood collection, all snakes were weighed (g), measured for snout-vent length (SVL in mm), and sexed. Females were gently palpated to determine gravidity. Snakes included juveniles and adults (SVL range: 202—820 mm) and did not differ in SVL between the regions ($t_{84} = 0.63$, $P = 0.53$). Body condition, a measure of size-corrected weight, is known to affect stress response in snakes (e.g., Moore et al., 2001; Palacios et al., 2012). However, such a metric cannot be applied to reproductive females as the developing mass of embryos confounds estimates of tissue energy stores, therefore precluding tests of the influence of body condition on the response of pregnant snakes. As the focus of this study is to examine the relationship among stress response variables within individuals and the potential flexibility of these systems across populations and sex categories, including pregnant females, we are not including body condition as a predictor variable in our models. Using males and non-pregnant females, body condition (calculated as the residual of the log-mass on log-SVL regression; Weatherhead and Brown, 1996), did not differ between the georegions ($t_{69} = -0.16$, $P = 0.88$).

2.2 Capture-Restraint Protocol

A subset of snakes (Table 1) was kept for repeated blood draws following a standardized restraint protocol, which elicits a glucocorticoid response in a variety of reptile and avian taxa (e.g., Moore et al., 2000a; Palacios et al., 2012; Romero, 2002). After collection of the

baseline sample, snakes were maintained in breathable nylon bags at ambient temperature and we collected a smaller amount of blood (20-30 μL) at 15 min, 45 min, 90 min, 3 h, and 6 h post-capture. After the 6 h bleed, snakes were transferred to plastic boxes (20 cm $\times$ 30 cm $\times$ 17 cm) with shredded paper bedding and a water dish, stored in a wooden hutch in the shade at ambient outdoor temperatures. We then collected blood in the mornings on the following three days, generally between 9 00 h and 9 30 h. This protocol resulted in a total of nine samples collected for each snake. Additionally, we prepared blood smears as above for a subset of snakes at 3 h and 3 d. After sample collection, all snakes were released at their point of capture. Fieldwork was conducted under permits from the California Department of Fish and Game and the Iowa Department of Natural Resources, and procedures were approved by the ISU Institutional Animal Care and Use Committee (protocol 1-12-7285-J).

2.3 Laboratory Methods

We measured CORT concentrations in blood plasma using double-antibody radioimmunoassay kits (Kit #07120102, MP Biomedical, Orangeburg, NY, USA) following the protocol in Robert et al. (2009) with samples diluted 1:80 to account for high concentrations in this study. This assay has been previously validated for use with fecal samples in this species (Halliday et al., 2015), which we confirmed with parallelism of a serially diluted pooled sample (heterogeneity of slopes: $F_{1,10}$: 1.20, $P = 0.30$). Measurements were made using two kits, with interassay variability measured using kit-provided controls (CV%: 8.80% and 11.80% for low and high controls, respectively). All samples were run in duplicate and accepted if CV% between the duplicates was less than 10%; otherwise, samples were re-run. We measured glucose from 1.5 μL of the thawed plasma using a FreeStyle Lite Glucometer (Abbott Diabetes Care, Alameda,

CA). We assigned a value of 20 mg/dL to samples which measured below the lower threshold of the glucometer (20 mg/dL; $N = 10$ samples). We quantified the relative abundances of lymphocytes, monocytes, heterophils, and basophils in blood smears following the procedure described in Sparkman and Palacios (2009). Briefly, we used a compound microscope to classify the first 100 leukocytes encountered under 1000 $\times$ magnification and then calculated the ratio of heterophils to lymphocytes (H:L ratio).

2.4 Statistical Methods

All analyses were conducted with program SAS version 9.4 (SAS Institute, Cary, NC). Graphics were created using ggplot2 (Wickham, 2009) in the programming language R (v. 3.1.2, R Core Team, 2014).

Repeated Measures

We assessed the effects of biotic and abiotic factors on the time series of CORT concentration, glucose concentration, and H:L ratios using a mixed-effects generalized linear model in PROC MIXED. The three dependent variables were log₁₀-transformed to meet assumptions of normality (Sokal and Rohlf, 2011). We began with models including all interactions among our three categorical predictor variables (georegion, sex category, and time point) and removed non-significant terms excepting the interactions of georegion and sex category with time point, which test our hypotheses of interest. The repeated-measures model for the analysis of dependent variables took the form:

$$Y = \mu + \text{Georegion} + \text{Sex Category} + \text{Time Point} + \text{Georegion} \times \text{Time Point} + \text{Sex Category} \times \text{Time Point} + \text{SVL} + \text{Julian Date} + \text{Population}(\text{Georegion}) + \varepsilon$$

where μ represents the grand mean and ε represents the error term. We also included individual as a repeated-measures random effect modeled with first-order ante-dependence [ante(1)] covariance structure, which accounts for asymmetric intervals between measurements (Littell et al., 2006). We estimated denominator degrees of freedom for random-effects model F -tests using the Kenward-Roger Degrees of Freedom Approximation (Kenward and Roger, 1997). Initial analyses indicated that baseline body temperature was not a significant predictor of any of the three dependent variables, so this effect was removed from subsequent models (CORT: $P = 0.27$; Glucose: $P = 0.51$; H:L ratios: $P = 0.48$). Additionally, baseline dependent variables did not differ significantly between years for the Iowa samples, so we pooled samples from 2013 and 2014 for analysis (CORT: $P = 0.090$; Glucose: $P = 0.086$; H:L ratios: $P = 0.66$).

Cumulative Measures: Total Response, Fold Increase, & Time to Baseline

We assessed the influence of the above factors on three cumulative measures of stress response for both CORT and glucose: total response, fold increase, and time to return to baseline. Total response represents the cumulative amount of hormone available for interaction with receptors or glucose available for use over the time in captivity and was calculated as the area under the curve from baseline to the final time point, using the absolute values of CORT and glucose concentrations. Romero (2004) advocates the calculation of stress-response measures from zero (rather than normalized to an individual's baseline) to provide a more biologically relevant estimate of the total actions of these molecules. Furthermore, our data include values over an

extended time period during which many individuals dropped below baseline levels in these measures, so including only data on values above the baseline for each individual would result in a significant loss of information. Our measures of total response, therefore, represent the cumulative physiological response over time in captivity, including variation in both starting values and duration of the stress response. Since animals were initially caught at different times of day, they spent slightly different amounts of time in captivity until their blood draw on day three. To normalize each of these measures to the same amount of time in calculating the total CORT and glucose responses (the area under the curve for the duration of animals' time in captivity), we censored these calculations at the lowest amount of time to the 3 d blood draw (3990 minutes from time of capture).

Fold increase, a measure of the relative increase of a physiological variable under stress condition compared to baseline, was calculated as the ratio of the highest value attained over the baseline value for that individual (Patterson et al., 2014). Log₁₀-transformed total and fold-increase measures were analyzed with an ANCOVA with the form:

$$Y = \mu + \text{Georegion} + \text{Sex Category} + \text{SVL} + \text{Julian Date} + \text{Population}(\text{Georegion}) + \varepsilon$$

where μ represents the grand mean and ε represents the error term.

Time to baseline is the number of minutes it took an individual to reduce its concentration of the hormone/metabolite to within two standard errors (calculated based on the set of individuals in the restraint protocol) of its own baseline value after reaching its maximum value. This measure

is thus an indicator of how long the hormone/metabolite concentration remained elevated during the restraint protocol. We calculated this time as the intercept of the line connecting the first measurement below this threshold (baseline + 2 SE) with the preceding measurement. Since this measure was censored at both extremes (some individuals never exceeded the baseline while some never returned), we used a non-parametric Wilcoxon-Mann-Whitney test to compare the rank-order of values between georegions using PROC NPAR1WAY.

Within-individual Correlations

We tested whether different physiological measures were correlated within individuals and how this relationship changed over time. We estimated the within-individual correlations of CORT concentration with glucose concentration and H:L ratios at the baseline, 3 h, and 3 d time points, using ordinary least-squares linear regressions conducted in PROC REG. Variables were $\log_{10}$ -transformed and normalized to mean of zero and unit variance so that effect sizes (slopes of relationships) could be compared directly among variables.

3. Results

3.1 Repeated Measures

For those samples for which we recorded exact time interval between first capture and first bleed (“bleed time,” $N = 66$), we confirmed that bleed times of ten minutes or less do not affect baseline CORT concentrations ($t_{64} = 1.51$, $P = 0.14$, $R^2 = 0.035$). However, we identified a significant effect of bleed time on baseline glucose concentrations ($t_{64} = 2.68$, $P = 0.0093$, $R^2 = 0.10$). In general, handling stress elevates glucose levels more rapidly than CORT concentrations likely due to the rapid effects of epinephrine or norepinephrine in increasing circulating

glucose (Sapolsky et al., 2000). While this trend was significant, glucose levels continued to increase across observations at subsequent time points (Fig. 1B), so this baseline increase exerted only a marginal effect on our analysis and did not change our results qualitatively. Nonetheless, we offer this as a precautionary note of the potential for rapid increase in glucose levels in squamate reptiles under handling stress.

Both plasma CORT and glucose concentrations, but not H:L ratios, were affected by time in restraint and the shapes of these response curves differed between georegions (Table 2, Fig. 1). At the first time point (“baseline”), CA snakes had higher CORT concentrations compared to IA snakes (difference of least-squares means, Tukey adjusted $P = 0.015$). While this is the only time point at which the pairwise difference between georegions for CORT concentration differed significantly, the significant georegion $\times$ time interaction demonstrates that the shapes of the curves differed between georegions. CORT concentration in CA snakes peaked and was significantly above baseline at 45 min (difference of least-squares means, Tukey-adjusted $P = 0.0099$). CORT concentration in IA snakes, however, elevated more rapidly and was significantly above baseline at 15 min and peaked at 90 min, with every time point from 15 min to 3 d significantly above baseline (all differences of least-squares means, Tukey-adjusted $P < 0.021$).

When taken across the entire time series, glucose concentrations were higher in snakes from IA (as demonstrated by a significant effect of georegion), with a significant pairwise difference at the 6 h time point (Tukey adjusted $P = 0.046$). The shapes of the glucose response curves differed between georegions, as demonstrated by the significant georegion $\times$ time interaction. Glucose concentration in CA snakes peaked at 45 min and was significantly elevated above

baseline between 15 min and 90 min (all differences of least-squares means, Tukey-adjusted $P < 0.025$). In IA snakes, glucose took longer to reach a maximum and stayed elevated for a longer period, peaking at 3 h and significantly above baseline between 15 min and 6 h (all differences of least-squares means, Tukey-adjusted $P < 0.001$). Additionally, we found significant heterogeneity among populations from each georegion in measures of glucose concentration and H:L ratios. No other effects significantly impacted these physiological measures.

3.2 Cumulative Measures: Total Response, Fold Increase, & Time to Baseline

Total CORT and CORT fold increase differed between georegions, but in opposite directions: CA snakes had higher total CORT response while snakes from IA showed a greater CORT fold increase (Table 3, Fig. 2). Total CORT and glucose fold increase declined with increasing snake size and there was a similar trend for both CORT fold increase and total glucose. Total glucose response was also reduced for snakes caught later in the year. Additionally, we found significant heterogeneity among populations within each region for glucose fold increase, largely driven by one CA population with a much reduced response compared to the other populations from this georegion. CA snakes tended to return to baseline CORT values generally faster than IA snakes (median time for CA snakes was 1708 min, while more than half of the IA snakes never returned to baseline; Wilcoxon-Mann-Whitney exact test, two-sided $P = 0.069$). This trend corroborates with our finding that the least-squares means in the repeated-measures models of CORT concentration were elevated above baseline in IA snakes for every time point through 3 d while CA were significantly different only at 45 min (see above). CA snakes returned to baseline glucose values faster than IA snakes (median times for CA and IA, respectively: 328 and 1162

min; Wilcoxon-Mann-Whitney exact test, two-sided $P = 0.0034$), again corroborating our results from the repeated-measures model of glucose concentration.

3.3 Within-individual correlations

Within individuals, baseline CORT concentration was positively correlated with both baseline glucose concentrations and H:L ratios. These measures were not correlated, however, after 3 h of the capture-restraint protocol. After 3 d, CORT concentration was again correlated with glucose concentration, though not with H:L ratios. The total amount of variation in glucose concentration and H:L ratios explained by CORT concentration was small, indicating that even as these measures are related within individuals there are likely other factors determining changes in each biomarker over time in response to a stressor (Table 4, Fig.3).

4. Discussion

In this widespread reptile species, we found that individuals from CA had higher baseline CORT levels than individuals from IA (Fig. 1A). A simple interpretation of this result alone might lead to the conclusion that snakes from CA maintain a more active state of the HPI axis relative to snakes from IA, either in response to a greater chronic stress load or differences in food availability. Our measures of additional baseline physiological markers, as well as measures while snakes experienced the short-term stress of captivity and restraint, suggest that the former interpretation is inaccurate. While CA snakes also mounted a greater total CORT stress response, they more rapidly reduced both CORT and glucose levels, generally associated with a healthy stress-response profile (Dickens and Romero, 2013; Tables 2-3, Figs. 1-2). On the other hand, IA snakes began with lower baseline CORT levels, but their stress response was proportionally

greater and they maintained these elevated levels for longer periods of time. The higher baseline CORT in snakes from high-elevation populations in CA is concordant with expectations of elevated baseline glucocorticoids in colder regions with a reduced active season (reviewed in Jessop et al., 2016). Taken together, these findings indicate that either strategy or capacity to maintain organismal function and energy balance in the face of environmental variation – be it stressful or beneficial – may vary across a species' range. Thus a comparison of a single biomarker may not provide sufficient information to assess relative physiological status. Furthermore, stress-induced glucose and H:L ratio profiles were significantly heterogeneous among populations within each georegion, lending support to the idea that geographic variation can exist at multiple scales and that local conditions influence individuals' perception and/or response to stress (Table 2).

In addition to heterogeneity between and within georegions, we found significant among-individual variation in physiological profiles. Size, which can be used to some extent as proxy for age in snakes (Bronikowski and Arnold, 1999; Sparkman et al., 2007), was negatively correlated with cumulative stress-response measures of CORT and glucose concentrations (Table 3). This is contrary to Moore et al. (2000a), who reported increased total CORT response in longer male *T. sirtalis* individuals and suggested that this could be due to reduced sensitivity to negative feedback of the HPI axis in these male snakes during the mating season. This pattern is also opposite that generally found in mammals and birds, where hormonal stress response increases with age (Creel et al., 2002; Homberger et al., 2015; Pavitt et al., 2016; Sapolsky et al., 1986). We see two primary explanations for the pattern of reduced total response in larger individuals in the present study: individual snakes may be altering the activation of this

endocrine stress response pathway across their lifespan, with younger snakes exhibiting increased HPI reactivity. Under this scenario, individuals alter their physiology within their lifetime in a predictable manner. This can be an advantageous switch in response to changing food availability or predation threat as an individual grows larger or a result of senescence. Alternatively, this observation could be due to the removal of maladapted individuals from the population (van de Pol and Verhulst, 2006) that suffer the negative consequences of an overreactive response. We also found evidence that seasonal cycles influence stress-induced total glucose, but not baseline levels. This could be caused either by individuals changing their energetic response to stress across the season or a result of reduced energetic capacity, likely due to a decrease in food availability. Independent of seasonal variation, reproductive status did not affect stress response in female snakes, which is somewhat surprising given the changes in energetic demands associated with embryonic development (e.g., Bonnet et al., 2002) and higher CORT concentrations of non-reproductive female garter snakes found in a previous study (Sparkman et al., 2014). Such reproductive allocation decisions are potentially regulated by other hormonal systems independent of the HPI axis, such as thyroid hormones (e.g., Bona-Gallo et al., 1980).

Understanding variation in the maintenance of organismal homeostasis is critical for establishing links between indicators, such as glucocorticoids, glucose, and leukocyte counts, and physiological status (Bonier et al., 2009; Breuner et al., 2008; Goessling et al., 2015). Within individuals, we found a significant positive correlation of CORT concentration with glucose concentration and H:L ratios at baseline. This relationship disappeared after 3 h, but partially returned by 3 d (CORT correlated with glucose, but not H:L ratios), indicating the effect of

restraint stress on these correlations (Table 4, Fig. 3). This suggests that under baseline conditions, hormonal, energetic, and immune systems are coordinated within the individual to maintain steady-state functionality. Under the stress of restraint, however, these functions can become quickly disassociated or respond on different time scales. Previous work with squamate reptiles indicates that neither snakes nor lizards demonstrate correlated responses of CORT and H:L ratios to captivity stress (Seddon and Klukowski, 2012; Sparkman et al., 2014). If the correlation among these physiological variables under baseline conditions does represent the integrated operation of various systems in maintaining homeostasis, it is noteworthy how quickly – within a matter of hours – these functions can become decoupled. Furthermore, these biomarkers appeared to partially re-align after several days, suggesting that these resilient systems can quickly return to pre-stress conditions. Furthermore, the small amount of variation explained by CORT concentration on either glucose concentration or H:L ratios suggests that, while the HPI axis may be driving some changes in these variables, other hormonal systems likely exhibit an effect as well.

Circulating glucocorticoids exert negative feedback on the HPA/HPI axis, which serves to reduce overexposure to these hormones and subsequent negative effects (Sapolsky et al., 2000). With reduced sensitivity to negative control, CORT concentrations could remain elevated and have potentially detrimental effects on physiology and fitness (Korte et al., 2005), though this conclusion is based largely on laboratory studies and not in wild populations (Dantzer et al., 2014). Furthermore, sustained elevation of CORT concentrations will likely impact long-term energy balance not only through the use of energy stores such as glucose but also through an increase in energetic expenditure and increased metabolic rate (DuRant et al., 2008; Preest and

Cree, 2008; Wack et al., 2012). We found a pattern of both CORT and glucose concentrations peaking within several hours and declining over the next 3 d in captivity, though not always to baseline levels. This is in general agreement with previous work in squamates, which found that CORT concentration in lizards returned to baseline 6 h after a CORT injection (*Sceloporus occidentalis*: DuRant et al., 2008) and within 24 h after handling stress (*Uta stansburiana*: Mills et al., 2008).

Interestingly, we found that snakes from CA had a greater cumulative CORT response through 3 d in captivity, though they also returned to baseline CORT more rapidly than snakes from IA, as indicated by both comparisons of least-squares means and the time-to-baseline measures (Tables 2-3, Fig. 1A). This was accompanied by a more sustained increase in stress-induced glucose concentrations in snakes from IA and a longer time to return to baseline glucose levels (Tables 2-3, Fig. 1B). The rapid reduction in CORT concentration after initial stress response, as seen in snakes from CA, suggests a well-integrated hormonal response. The sustained elevation of both CORT and glucose concentrations we observed in snakes from IA may be a result of “wear and tear” on an overused hormonal system (sensu McEwen and Wingfield, 2010; Romero et al., 2009) and may indicate the populations are under chronic stress (Dickens and Romero, 2013). Given our experimental design, however, we cannot distinguish between the effective action of negative feedback on the HPI axis and differences in the perception of a stressor. CA snakes may more quickly acclimate to captive conditions than snakes from IA and therefore reduce the stress response over time, independent of the negative feedback exerted directly by CORT itself. In either case, clearly differences exist between snakes from different georegions in their negative feedback capacity, perception of stressors, and/or life-history allocations, all of which can have

important behavioral and long-term energetic consequences (Korte et al., 2005). We note a limitation of our experimental design, imposed by logistical constraints, is that CA snakes were sampled over a relatively brief portion of the active season. While we have statistically controlled for the effects of Julian date in our comparison of georegions, we note that IA snakes were sampled across a longer season and therefore from a broader range of immediate environmental conditions.

We see two important avenues for future research in this area. First, examining the causes and consequences of among-individual variation in stress response profiles on performance and reproduction will be key to linking hormonal responses with fitness (Bonier et al., 2009; Breuner et al., 2008; Cockrem, 2013; Cox et al., 2016). Given the complex mechanisms by which these snakes maintain physiological functionality, data linking hormonal profiles with survival and/or reproductive output, for both males and females, is essential to describing how future environmental variation will affect population dynamics. Second, we need more data explaining the within-individual mechanisms determining variation in response to stressors. For example, the CORT response to stress may be regulated at a number of levels, such as corticosteroid-binding globulin (Breuner et al., 2013; Edwards and Boonstra, 2015), receptor density (Lutterschmidt and Maine, 2014; Romero, 2002), or by the sensitivity of the adrenals to activation by HPA/HPI axis (Dayger and Lutterschmidt, 2016). By demonstrating both variation across geography and the coordination of stress-response biomarkers within individuals, our results provide key insights into the complex physiological regulators of stress response systems in vertebrates and point to future directions for quantifying the consequences of variation in this stress response.

Acknowledgements. For assistance with fieldwork, we thank N. Carver, K. Kauffman, K. Martin, A. Pottebaum, C. Rodriguez, & S. Zylstra. Thanks to R. Polich and R. Telemeco for assistance with labwork and C. Vleck for the use of lab space and equipment. G. Burghardt, B. Danielson, L. Hoekstra, and F. Janzen provided constructive comments on the manuscript and F. Miguez offered statistical advice. Access to field sites was generously granted by W. Clark, B. & T. Danielson, D. Sheeley with Polk County Conservation, Oakridge Research & Education Prairie, T. Rickman with the U.S. Forest Service, and the IA Department of Natural Resources. This project received funding support from the Iowa Science Foundation (13-07), IA Department of Natural Resources Wildlife Diversity Program, Prairie Biotic Research Inc., the Society for Integrative and Comparative Biology Grants-in-Aid of Research program, and the National Science Foundation (IOS-0922528 to AMB). EJG received additional support from a fellowship from the Iowa State University Office of Biotechnology.

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Number of individuals for baseline and (restraint) procedures						
Georegion	Populations sampled	Year	Males	Non-gravid females	Gravid females	Total
California	4	2013	17 (6)	8 (2)	10 (2)	35 (10)
Iowa	4	2013	13 (8)	10 (7)	5 (1)	28 (16)
Iowa	4	2014	16 (3)	7 (3)	0 (0)	23 (6)
Total			46 (17)	25 (12)	15 (3)	86 (32)

Table 1. Individual *Thamnophis sirtalis* included in this study. Top numbers indicate baseline samples; numbers in parentheses indicate individuals included in restraint protocol.

		CORT (<i>N</i> = 329 samples) ^a	Glucose (<i>N</i> = 334 samples) ^a	H:L Ratio (<i>N</i> = 83 samples) ^b
<i>Time</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>)	9.93 (8, 57.5)	17.11 (8, 58.2)	0.98 (2, 21.7)
	Pr > F	< 0.0001***	< 0.0001***	0.39
<i>Georegion</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>)	0.15 (1, 115)	9.90 (1, 75.8)	0.18 (1, 53.8)
	Pr > F	0.70	0.0024**	0.67
<i>Sex Category</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>)	0.02 (2, 50.4)	0.73 (2, 40.4)	0.53 (2, 36.1)
	Pr > F	0.98	0.49	0.59
<i>SVL</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>)	1.19 (1, 52.3)	2.10 (1, 58.2)	0.97 (1, 53.1)
	Pr > F	0.28	0.15	0.33
<i>Population(Georegion)</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>)	0.43 (6, 94.6)	3.34 (6, 86)	2.37 (6, 55.4)
	Pr > F	0.86	0.0053**	0.042*
<i>Julian Date</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>)	1.13 (1, 108)	2.80 (1, 89.5)	0.10 (1, 44)
	Pr > F	0.29	0.098	0.75
<i>Georegion</i> × <i>Time</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>)	2.74 (8, 60)	2.26 (8, 61.5)	1.80 (2, 22.3)
	Pr > F	0.012*	0.034*	0.19
<i>Sex Category</i> × <i>Time</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>)	0.84 (16, 80.6)	2.62 (2, 35.2)	2.28 (4, 22.7)
	Pr > F	0.64	0.42	0.092

Table 2. Results of repeated-measures mixed model analysis of log₁₀-transformed plasma CORT concentration, glucose concentration, and H:L ratios in wild-caught *Thamnophis sirtalis* from IA and CA. Individual was treated as a repeated-measure random effect and denominator degrees of freedom for *F*-tests were calculated using the Kenward-Roger Degrees of Freedom Approximation (Kenward and Roger, 1997). Significant effects shown in bold with one (P < 0.05), two (P < 0.01), or three asterisks (P < 0.0001). SVL = snout-vent length (mm).

^aNumber of samples is reduced for CORT and glucose due to plasma limitations.

^bH:L ratios were measured in a subset of samples from baseline, 3 h, and 3 d timepoints.

		Total CORT^a	CORT fold increase	Total Glucose	Glucose fold increase
<i>Georegion</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>) Pr > F	5.21 (1, 22) 0.033*	5.72 (1, 23) 0.025*	2.69 (1, 23) 0.11	0.48 (1, 23) 0.50
<i>Sex Category</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>) Pr > F	0.38 (2, 22) 0.69	1.63 (2, 23) 0.22	1.64 (2, 23) 0.22	1.90 (2, 23) 0.17
<i>SVL</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>) Pr > F	6.58 (1, 22) 0.018*	3.77 (1, 23) 0.065	4.27 (1, 23) 0.050*	7.48 (1, 23) 0.012*
<i>Population(Georegion)</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>) Pr > F	1.20 (3, 22) 0.33	0.56 (3, 23) 0.65	1.17 (3, 23) 0.34	3.71 (3, 23) 0.026*
<i>Julian Date</i>	<i>F</i> (<i>df_n</i> , <i>df_d</i>) Pr > F	2.70 (1, 22) 0.11	0.61 (1, 23) 0.44	8.25 (1, 23) 0.0086**	1.97 (1, 23) 0.17

Table 3. ANCOVA results for analysis of log₁₀-transformed cumulative measures of stress-induced plasma CORT concentration and glucose concentration in wild-caught *Thamnophis sirtalis* from IA and CA. Significant effects shown in bold with one ($P < 0.05$) or two ($P < 0.01$) asterisks. SVL = snout-vent length (mm).

^aN = 32 individuals for all cumulative measures except Total CORT, for which one individual was excluded due to missing data at the final time point.

Time	Measure	N	R ²	Pr > t
Baseline	Glucose Concentration	86	0.073	0.012*
	H : L Ratio	53	0.12	0.0093**
3 h	Glucose Concentration	31	0.010	0.59
	H : L Ratio	14	0.0074	0.76
3 d	Glucose Concentration	31	0.13	0.045*
	H : L Ratio	16	0.058	0.17

Table 4. Within-individual correlations of plasma CORT concentration with glucose concentration and heterophil : lymphocyte (H : L) ratios at three time points in wild-caught *Thamnophis sirtalis*. All variables were log₁₀-transformed and normalized to mean of zero and unit variance before analysis (see text for details). Significant relationships shown in bold with one ($P < 0.05$) or two ($P < 0.01$) asterisks.

Figure 1. Back-transformed least-squares means of baseline plasma CORT concentration (Panel A), glucose concentration (Panel B), and heterophils : lymphocyte (H : L) ratios (Panel C) across 3 d in captivity, shown with fitted loess curve. Data are from wild-caught *Thamnophis sirtalis* from IA and CA. Asterisks denote significant pairwise differences in least-squares means between georegions at a given time point (see text for statistical details). Error bars represent $\pm$ SE. Note change in x-axis scale after 360 min.

Figure 2. Back-transformed least-squares means of cumulative CORT (Panels A-B) and glucose (Panels C-D) measures by georegion for wild-caught *Thamnophis sirtalis* from CA and IA. Significant differences between georegions ($P < 0.05$) of $\log_{10}$ -transformed variables are shown with an asterisk (see text for statistical details). $N = 32$ individuals for all cumulative measures except Total CORT, for which one individual was excluded due to missing data at the final time point. Error bars represent $\pm$ SE.

Figure 3. Scatterplots of within-individual relationship between plasma CORT concentration and plasma glucose concentration and heterophil : lymphocyte (H : L) ratios at baseline (Panels A-B), 3 h (Panels C-D), and at 3 d (Panels E-F) for wild-caught *Thamnophis sirtalis*. Regression lines shown for significant relationships of $\log_{10}$ -transformed and normalized variables (see text for statistical details).